Uptake of Lipids by the Entomophilic Nematode Romanomermis culicivorax

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Abstract: *Romanomermis culicivorax* juveniles were dissected out of *Aedes aegypti* larvae 7 days after infection and incubated under controlled conditions in isotonic saline containing a ^{14}C-labeled fatty acid (palmitic acid), monoacylglycerol (glycerol monoolein), or triacylglycerol (glycerol tripalmitate) nutrient source. The mermithid absorbed each of these lipids from the incubation medium, the rate of uptake being greatest for glycerol monoolein. No lipase activity was detected in whole nematode homogenates or in the media in which the nematodes were incubated. It is suggested that the nematode transports complex lipid molecules across its outer cuticle intact. *Key words: Aedes aegypti,* fatty acid, lipase, mermithid, monoacylglycerol, nutrition, triacylglycerol.

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The juvenile stages of the mermithid nematode *Romanomermis culicivorax* (Enoplida: Mermithidae) develop in the hemocoel of larval mosquitoes. In common with other mermithids, *R. culicivorax* feeds only during the parasitic phase of its life cycle. The nutrition of mermithids is considerably modified from the basic nematode pattern. The parasitic juveniles absorb nutrients from the host's hemolymph across their outer cuticle and store them in a storage organ (trophosome) for subsequent utilization by the free-living stages. In *R. culicivorax,* lipids constitute the predominant storage metabolite (9). Gordon et al. (6) showed that the trophosomal lipids of *R. culicivorax* and the blackfly-parasitic mermithid, *Neomesomermis flumenalis* (= *Mesomermis flumenalis*), comprise in order of prevalence: triacyglycerols, phospholipids, sterol esters, and free sterols. Stored lipids may be metabolized for energy by the postparasitic juveniles and adults, which possess a functional β-oxidation pathway (8).

Despite the importance of lipids as a food store in *R. culicivorax,* no studies have been done for any mermithid species to ascertain what kinds of dietary lipids the nematode(s) requires from the host's hemolymph. Accordingly, attempts to culture *R. culicivorax* in vitro have met with limited success, because the culture media (and consequently, the trophosomes of the developing nematodes) were deficient in lipids (4).

Saponifiable lipids are present in the hemolymph of the larval *Aedes aegypti,* a laboratory host for *R. culicivorax,* mainly as triacylglycerols, monacylglycerols, and free fatty acids (7). The present study was done to determine which of these lipid categories constitute a nutrient source for the parasitic *R. culicivorax.* Since it has been suggested (14,15) that parasitic juveniles of mermithids secrete enzymes to predigest host nutrients before absorbing them, a second objective was to investigate whether the nematode secretes lipase(s) to effect hydrolysis of complex hemolymph lipids.

MATERIALS AND METHODS

Source of biological material: A laboratory colony of *R. culicivorax* was maintained by propagating the mermithid through *A. aegypti* larvae. The method employed was a scaled-down version of the mass-rearing system developed by Petersen and Willis (11); the nematode's adult and egg stages were stored (20 C) in moist autoclaved sand.

Developing nematode juveniles required for lipid uptake and lipase studies were obtained by infecting newly hatched *A. aegypti* larvae with controlled doses of *R. culicivorax* and maintaining infected hosts at 27 C (1). Under these conditions postparasitic nematodes were found to emerge from the host 8–10 days after infection. Infected mosquitoes were dissected 7 days after infection in half-strength Dulbecco's phosphate buffered saline (½ DPBS; Grand Island Biological Company, New York, USA) and nematodes removed.

Uptake of lipids: Emulsions of radiolabeled lipids were prepared by vigorous

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manual shaking in ½ DPBS. Groups of 50 nematodes were transferred to covered glass dishes (3 cm diam) and incubated (27 C) in 1 ml of the saline containing the requisite amount of the radiolabeled lipid precursor. (Radiochemicals were obtained from Amersham/Searle Corporation, Canada, and Applied Science Laboratories, Inc., USA.) Uptake of lipids by the nematodes was separately measured for three major types of lipids: (i) *free fatty acids:* palmitic acid-U-^{14}C (403 μCi/μmole); (ii) *monoacylglycerols:* glycerol monoolein-1-^{14}C (58 μCi/μmole); and (iii) *triacylglycerols:* glycerol tripalmitate-1-^{14}C (57 μCi/μmole).

Nematodes were incubated according to one of two regimens: (i) for 1 h with 2.15×10^{-3} μmoles isotope; or (ii) for 6 h with 2.15×10^{-2} μmoles isotope. At the end of the incubation period, the nematodes were washed free of radioactive medium in three consecutive washes (1-min, 2-min, 2-min duration) with 10 ml ½ DPBS. Assay for radioactivity of a 0.1-ml sample of the third wash using a liquid scintillation counter showed counts only slightly (< 50 cpm) higher than background. Washed nematodes were oven-dried (60 C), weighed on a Cahn Electrobalance (Model 21), then stored frozen (–20 C) in ½ DPBS until analysis. The groups of 50 worms were transferred to scintillation vials and dissolved at 60 C in 0.4 ml of 'NCS' solubilizer (Amersham/Searle). The contents of each vial were taken up in 13 ml 'Aquasol' liquid scintillation cocktail (New England Nuclear Corp., Boston, Mass., USA) and acidified with 0.5 ml 1 N hydrochloric acid.

Lipase determinations: Two procedures were used to investigate whether the parasitic juveniles synthesized/secreted a lipase:

(i) *Colorimetric.* Two hundred nematodes were homogenized in 0.5 ml ½ DPBS. The homogenate was centrifuged (4 C, 20 min, 700 g), and 0.1 ml aliquots of the supernatant incubated (10 min, 30 C) with a buffered (pH 8.5) suspension of olive oil. The free fatty acids were then separated in the form of their copper salts by chloroform extraction and the copper colorimetrically determined with diethyldithiocarbamate (17).

(ii) *Radioisotope procedure.* A method was devised, based on the capacity of lipase(s) to effect the hydrolysis of a radiolabeled triacylglycerol substrate into radiolabeled fatty acids. "Test" systems consisted of groups of 20 parasitic juveniles, incubated (20 C) in covered glass dishes (3 cm diam) containing 1 ml ½ DPBS together with 0.5 μCi glycerol tripalmitate-1-^{14}C, held in emulsion. "Control" dishes containing only saline and isotope were incubated alongside the "Test" systems, as were "Standards" dishes containing only saline, isotope, and 100 enzyme units of pancreatic lipase (E.C. No. 3.1.1.3; Sigma Chemical Co., St. Louis, Mo., USA). At the end of 5-min and 60-min incubations, nematodes were removed from the test samples. Incubation media were vortexed (1 min) with 1 ml chloroform to extract lipids. The upper layer was discarded and the lower (lipid-containing) layer concentrated to ca. 50 μl by evaporating the chloroform in a stream of N_2. The whole extract was spotted on a heat-activated TLC-plate (Silica Gel G); lipid fractions were then separated according to the procedure of Mangold (10) and visualized with I_2 vapor. Areas of plate-coatings corresponding in size and position to those of fatty acid standards were scraped into scintillation vials along with 13 ml 'Omnifluor' liquid scintillation cocktail (New England Nuclear).

All samples (lipid uptake and lipase determinations) prepared for liquid scintillation counting were counted using a Beckman LS-3150T Liquid Scintillation Counter. Samples pertaining to uptake of lipids by the nematodes were corrected for quenching and counting efficiency by adding a ^{14}C-toluene internal standard.

RESULTS AND DISCUSSION

The parasitic juveniles of *R. culicivorax* were able to absorb each of the three types of lipids across their outer cuticles when the concentration of isotope was 2.15×10^{-2} μmole/ml and the time available was 6 h (Table 1). Under less optimal conditions (1 h incubation time, lower isotope concentration), however, uptake of glycerol tripalmitate by the nematode was seen to be extremely low in comparison to the other two isotopes.

No evidence of lipase activity was de-

Table 1. Uptake of various lipids by *Romanomermis culicivorax* parasitic juveniles.

^{14}C-isotope	Incubation time (h)*	Specific activity μmoles $\times 10^{-7}$/ mg dry wt.†
Palmitic acid	1	154.6 ± 15.4
	6	349.9 ± 33.0
Glycerol monoolein	1	314.4 ± 10.8
	6	2,496.9 ± 103.0
Glycerol tripalmitate	1	10.6 ± 2.9
	6	180.2 ± 15.2

*Isotope concentration was 2.15×10^{-3} μmoles/ml for all 1-h incubations and 2.15×10^{-2} μmoles/ml saline for all 6-h incubations.

†Values are means ± S.E. of four replicates, each consisting of 50 parasitic juveniles.

tected in nematode homogenates or in the medium in which the nematodes had been incubated. Zero absorbance values were obtained when four nematode homogenates were assayed colorimetrically for lipase activity. However, when a standard solution of pancreatic lipase (Sigma E.C. No. 3.1.1.3; 10^3 enzyme units/liter) was substituted for the homogenate, consistently high absorbance values were obtained. When the triacylglycerol (glycerol tripalmitate-1-^{14}C) substrate was incubated with pancreatic lipase, radiolabeled fatty acids were generated in levels that were readily detectable after thin layer chromatography (TLC) separation (Table 2). It would appear that hydrolysis of the triacylglycerol was completed during the first 5 min of the incubation period, since free fatty acid radioactivity levels after 60-min incubations were not significantly higher than they were after 5 min. After 60-min incubations, almost 20 percent of the radioactivity of the various fractions that separated out by TLC (free fatty acids, monoacylglycerols, diacylglycerols, triacylglycerols) was in the form of fatty acids. There was no increase in the fatty acid content of the medium, however, due to the presence of the nematodes. A relatively low level of radioactivity was recorded from TLC scrapings, corresponding in position to the fatty acid fraction, even when no nematodes or enzyme was present in the medium.

Comparing, on a mole-for-mole basis, data of equivalent incubation times, it is apparent that the monoacylglycerol was taken up more rapidly by the nematode than were the triacylglycerol or the simple fatty acid nutrient sources (Table 1). This fact, together with the apparent absence of lipase activity in the worms, indicates that the nematode transports complex lipid molecules across its cuticle intact, a characteristic which typifies cestodes more so than nematodes (3). However, the transport system(s) used by the nematode for transcuticular uptake of complex lipids must be quite different from those used by cestodes, which depend upon the formation of bile salt micelles in the host's intestine (2).

This study disproves, at least for mermithid parasites of mosquitoes, the hy-

Table 2. Assay for lipase activity in *Romanomermis culicivorax:* fatty acid determinations of incubation media.

Incubation system*	Time of incubation (min)	No. of replicates	Radioactivity of fatty acid fraction (cpm)†	Fatty acids: % of total radioactivity‡
Controls	5	5	11,372 ± 1,284	1.4 ± 0.2
	60	6	9,740 ± 1,000	1.4 ± 0.1
Standards	5	5	96,759 ± 15,623	14.5 ± 2.3
	60	5	107,470 ± 27,220	18.6 ± 4.7
Test	5	4	9,022 ± 1,985	1.3 ± 0.3
	60	4	7,336 ± 2,029	1.3 ± 0.3

*Test systems contain 20 nematodes, saline, and glycerol tripalmitate-1-^{14}C; standards contain saline, isotope, and pancreatic lipase; controls contain only saline and isotope.

†Means ± S.E. of radioactivity of the free fatty acid faction in the incubation medium separated out by TLC.

‡Radioactivity of the fatty acid (mean ± S.E.) fraction expressed as a percentage of the total radioactivity of all the lipid fractions that separated out by TLC.

pothesis advanced by Rubtsov (14,15) that mermithids predigest host nutrients by secreting digestive enzymes from cells of their longitudinal cords. There is some evidence (18) that mermithid parasites of chironomids may secrete hydrolytic enzymes (including lipase), but insufficient experimental details were provided by the authors to enable an assessment of their data.

Mermithids may vary in their capacity to transport complex molecules across their tegument. Ultrastructural studies have shown that the cuticle of *R. culicivorax* is traversed by pores and that a relatively large molecule such as the metalloprotein ferritin can be transported across it (12,13). However, the cuticle of *Mermis nigrescens*, parasitic in locusts, is nonporous (19) and incapable of absorbing dipeptides, proteins, or disaccharides (5,16).

The findings in this study suggest that progress may be made in the in vitro culture of *R. culicivorax* by supplementing potential culture media with both simple and complex lipids.

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